

Detection and monitoring of chronic anaemia, i.e. functional iron-deficiency anaemia. New approaches to improved treatment of patients with malignant diseases or kidney disorders.

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1. ***Introduction and background: “a touch of history”***

Most patients suffering from chronic renal failure or a malignant disease develop chronic anaemia. In order to avoid long-term negative consequences for the patient, it is essential that he or she obtains an adequate supply of iron.

In this context, both scientists and physicians have been interested in trying to understand the interaction between iron, relative iron deficiency and erythropoietic response in patients with anaemia.

■ *Iron deficiency (the way to “functional iron-deficiency anaemia”)*

Twenty years ago, Finch (1) summarised the results of several clinical investigations carried out on patients suffering from hereditary haemolytic anaemia or haemochromatosis. The clinical conditions which have been described can be considered kind of a “quasi-natural” experiment in relation to the iron-dependent dynamics of erythropoiesis.

- In healthy people with a basal erythropoietic metabolism, plasmatic iron-turnover is only slightly affected (as an indicator of the erythropoietic reaction of bone marrow), whereas transferrin saturation can range from very low to very high concentrations.
- In contrast, the erythropoietic reaction of patients with congenital haemolytic anaemia, (in which erythropoiesis can be up to six times the basal metabolism (2)), is adversely affected and limited by the level of the serum iron concentration, as well as by transferrin saturation (3).
- Patients with haemochromatosis undergoing multiple venesections showed an elevated erythropoietic response which is up to eight times the basal metabolism, having been attributed to the exceptionally high serum iron concentrations, as well as the high level of transferrin saturation (4). Healthy individuals, however, seemed to have difficulties to supply sufficient iron as soon as the basal erythropoietic metabolism was increased by more than a factor of 3 (5).

On the basis of these observations, Finch coined the term *relative iron deficiency*. Iron deficiency appears when erythrocytic iron demand exceeds the availability of iron, as well as the rate of making iron available, although sufficient stored iron was available (6).

Fig. 1: Schematics of Normal Iron Metabolism

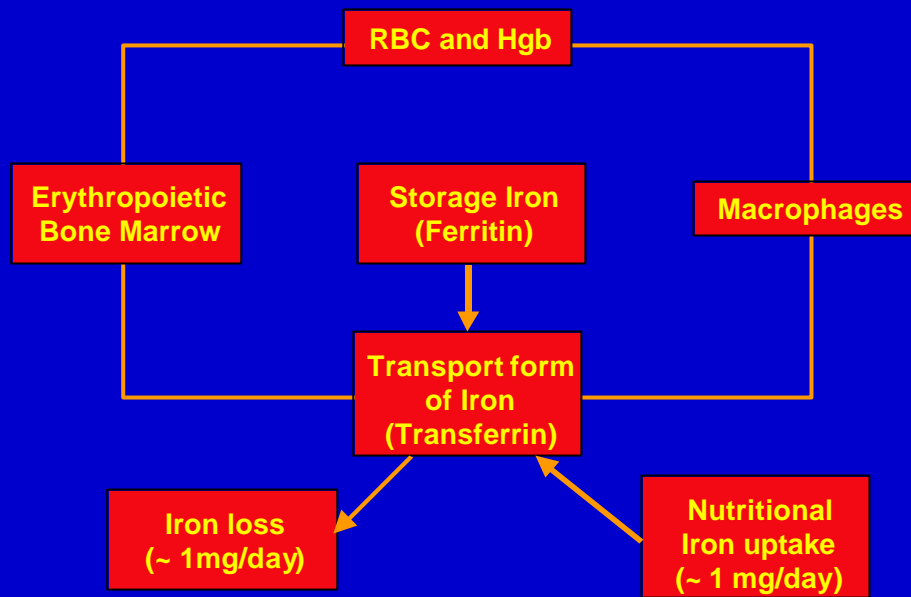


Fig. 2: Feed-back Controlled Circuit of Erythropoietin Production in Healthy Individuals

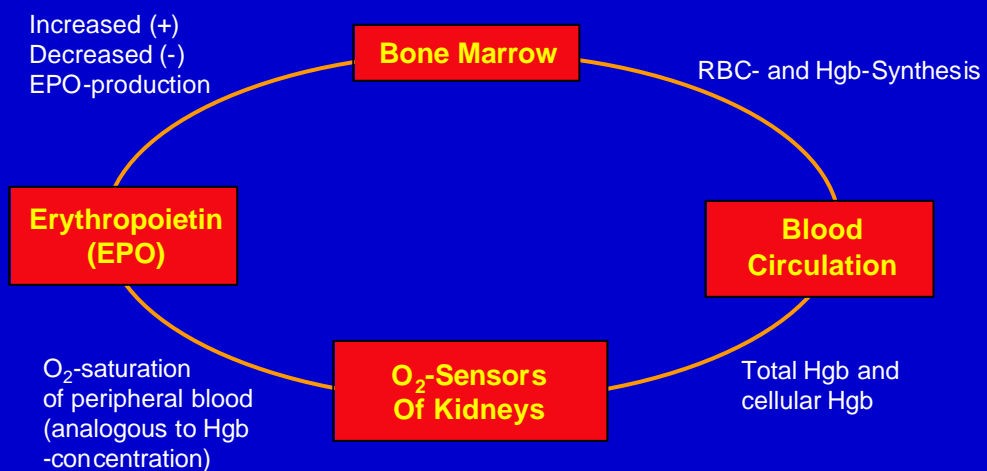
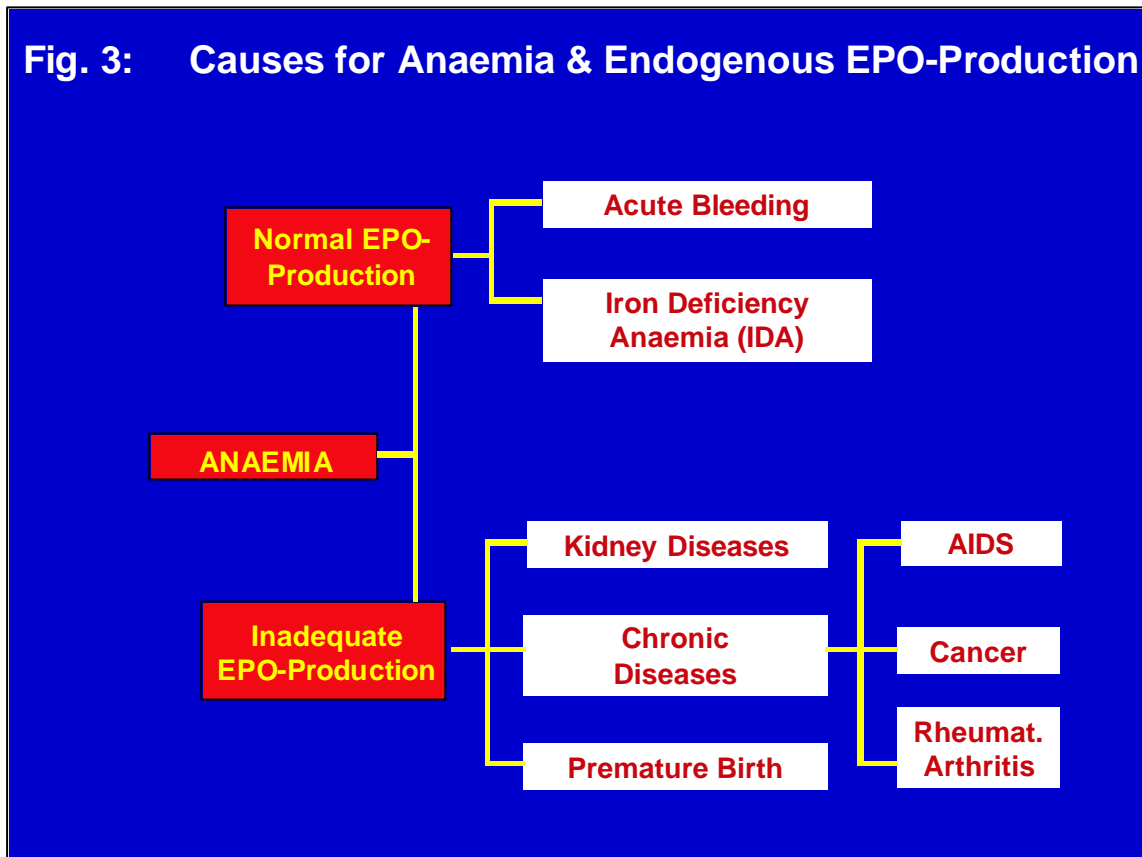


Fig. 3: Causes for Anaemia & Endogenous EPO-Production



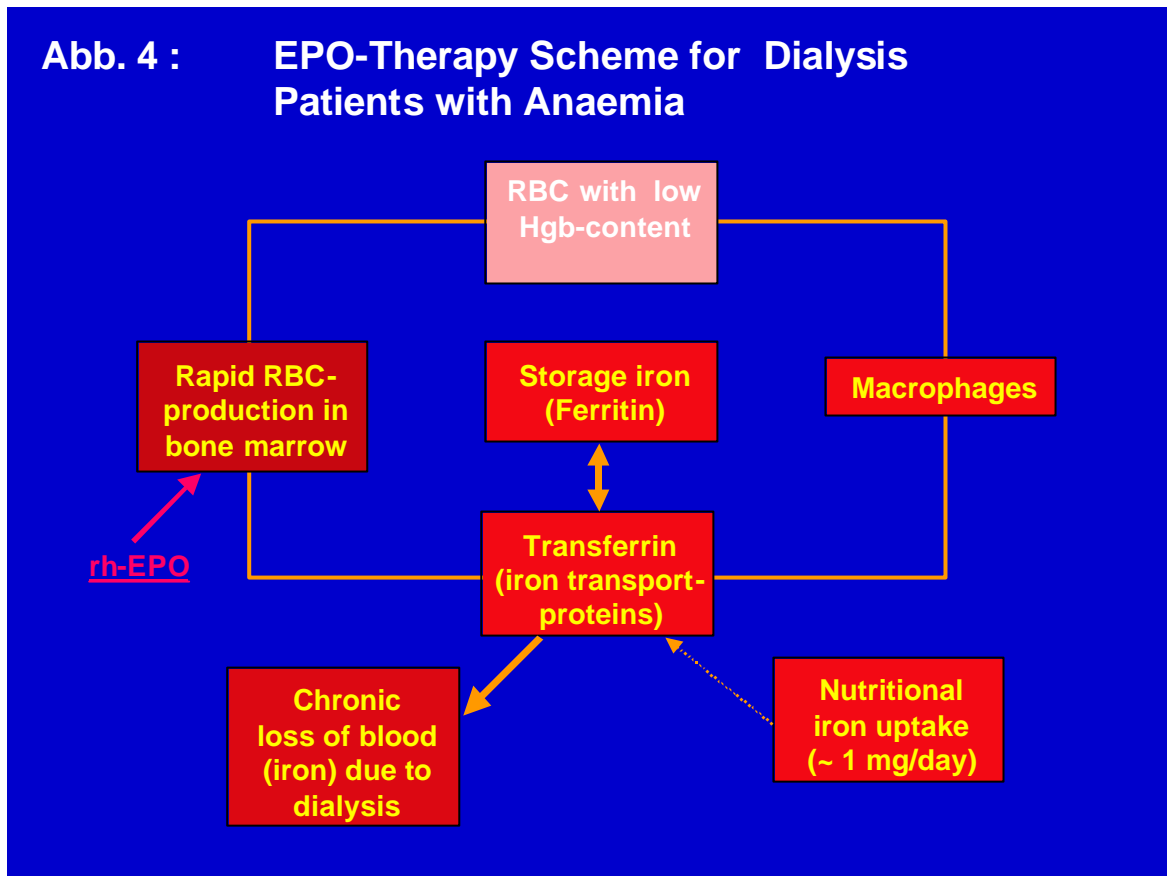
■ *Findings from studies of patients undergoing dialysis*

Above all, the success of treating anaemia caused by chronic renal failure with erythropoietin has increased our understanding of the interactions between erythropoiesis and iron metabolism (7,8).

In approximately 85% of dialysis patients suffering from anaemia, MCV still remains normal, which can be considered a distinct feature of this form of anaemia. In contrast, 96% of these patients show a distinct hypochromia (9).

A frequent complication observed with these patients is an inadequate response to administering erythropoietin. This can be caused by a series of co-morbid factors; in particular, aluminium intoxication, as well as, iron deficiency (10, 11).

Abb. 4 : EPO-Therapy Scheme for Dialysis Patients with Anaemia



Dialysis patients suffering from anaemia can also respond sub-optimally to oral iron therapy. The absorption rate in dialysis patients is by all means comparable with that of healthy individuals (12). However, patients undergoing erythropoietin therapy, can show an increased uptake of up to five times (13).

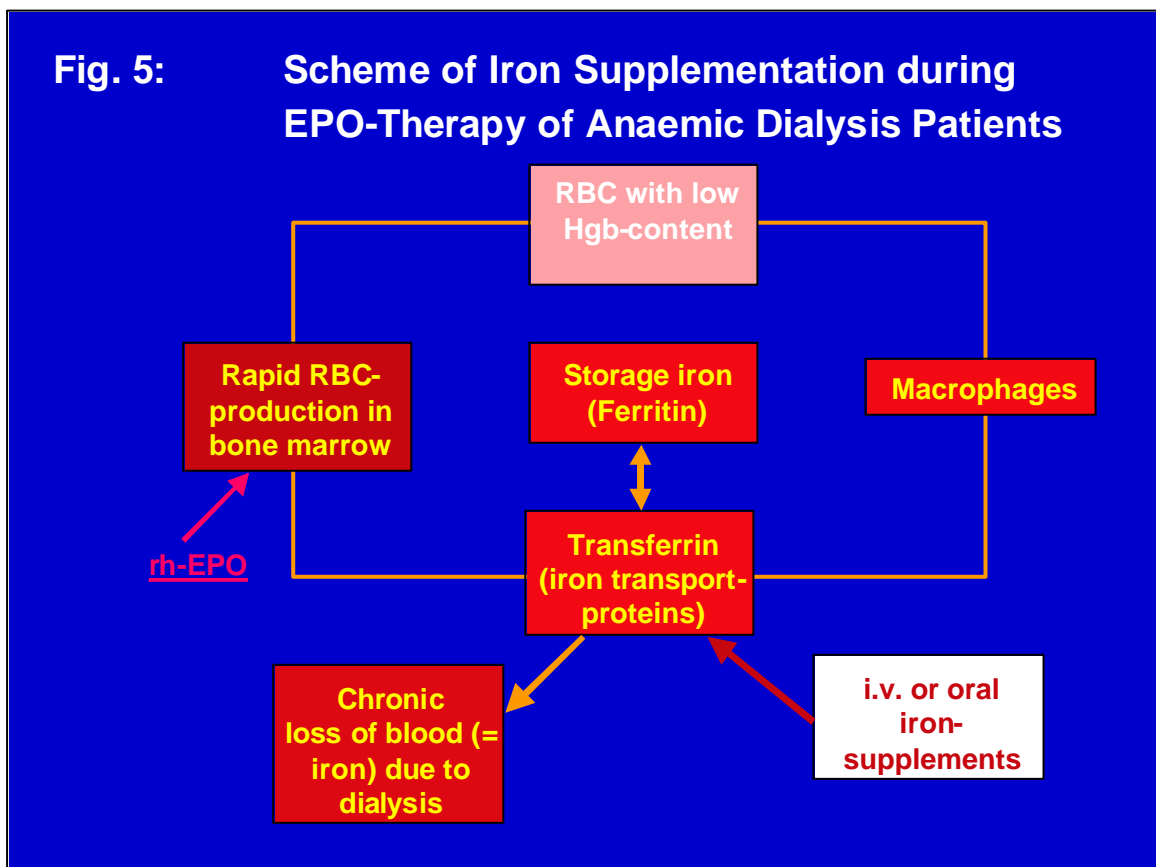
Nevertheless, the external loss of iron, which also includes the loss caused by haemodialysis and during blood tests, exceeds the gastrointestinal absorption capacity (7). Another problem is induced by inadequate therapy discipline, i.e. compliance, because of occurring gastrointestinal problems. In some cases, newer iron preparations have worse absorption rates, because of their galenism (14). Erythropoiesis with relative iron-deficiency becomes apparent if vitamin C is supplemented, in order to release iron from the reticulo-endothelial stores. The response of erythropoiesis will then be positive (11). The same is also true for the successful administration of intravenous iron with a simultaneous reduction of the supplied erythropoietin concentration (8).

■ *Significance of anaemia for dialysis patients*

Since anaemia constitutes a significant parameter in relation to the life expectancy of dialysis patients suffering from a chronic disorder (9, 15), intravenous iron therapy has become the standard therapy for most patients requiring erythropoietin (16).

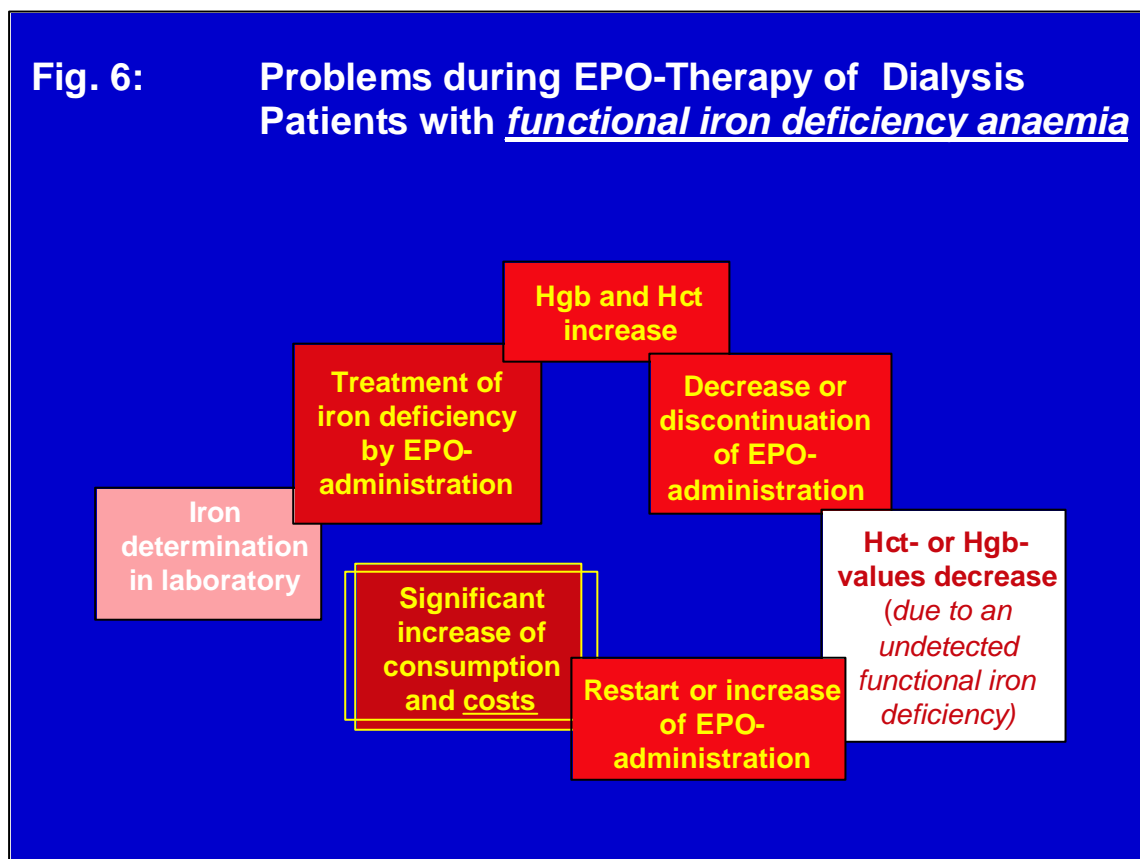
This combination therapy has also resulted in a considerable reduction in the erythropoietin administered, (with 100 mg iron administered twice a week intravenously, the erythropoietin dose is reduced by about 46 %, in order to maintain haematocrit values between 30 and 34 %). This erythropoietin dose-reduction is reported in comparison to a control-group undergoing oral iron-supplementation (8).

A further study, carried out on patients with chronic renal failure but not undergoing dialysis (17), demonstrated that two thirds of these patients did not react to iron supplementation administered orally, whereas they reacted well to intravenous administration given each week. This improvement was achieved despite initially high values of ferritin (up to 400 µg /L) (18). This shows that biochemical parameters are not appropriate for detecting erythropoiesis with concomitant relative iron deficiency.



In the meantime, in patients suffering from kidney disorders requiring erythropoietin, the intravenous administration of iron has become the preferred alternative over oral therapy. The clinical reaction to intravenously administered iron can be explained in terms of a complementary effect of erythropoietin-dependent release of iron from the reticuloendothelial system and the incorporation of it into the erythrocytic precursor cells (19).

The existence of an adversely affected erythropoiesis with concomitant relative iron deficiency can be referred to as a *functional iron-deficiency anaemia*. A “try and error approach” is still frequently used with dialysis patients suffering from chronic anaemia: cyclic reactions are the result in these patients following the pattern described below:



This constitutes an inadequate burden for the patients, as well as, uneconomic treatment with expensive medication. The *clarification of a functional iron-deficiency anaemia before as well as during erythropoietin therapy* (monitoring) with concomitant intravenous administration of iron is absolutely essential.

2. **At present, laboratory parameters for determination of iron status and erythropoiesis are of limited use**

Serum ferritin

As mentioned above, serum ferritin represents a parameter useful for the detection of stored iron, but not for iron availability. Low concentrations of serum ferritin in chronic renal failure are extremely variable and cannot be defined. Furthermore, in case of liver disease and inflammatory processes, there may be an unusual increase of serum ferritin observable.

Eventually, as a “rule of thumb”, one should assume that patients with ferritin levels below 200 µg/L will most probably react positively to the intravenous administration of iron.

Transferrin saturation

This is a parameter for defining the amount of circulating plasma iron relative to the total iron-binding capacity (TIBC). However, this parameter is characterised by a high degree of “within-day variation”. Additionally, it is a laborious and costly test procedure.

Erythrocyte ferritin and zinc protoporphyrin

Although some clinical studies have suggested using *erythrocyte ferritin* instead of serum ferritin as the reference parameter for patients with chronic illnesses suffering from anaemia, this parameter appears to be of limited value: it is both difficult to carry out and it is very insensitive towards dynamic changes. Only when almost the entire population of red blood cells has been replaced by iron deficient erythrocytes (and consequently low haemoglobin content) does this parameter decrease to pathologically low values.

The same also applies for determining zinc protoporphyrin (ZnP). In this case, as well, the majority of red blood cells must have been produced under iron deficiency, in order to reach pathological levels. Furthermore, ZnP is also susceptible in the presence of certain therapeutics and other plasma components. Therefore, ZnP does not provide great benefits to the detection of functional iron-deficient conditions.

3. Recent laboratory parameters for determining iron status and erythropoiesis

■ Erythrocyte indices

Dialysis-patients undergoing erythropoietin treatment show a progressive development of hypochromic, microcytic red blood cells (20). If more than 10 % of all erythrocytes belong to this population, (normal values < 2.5 %), one may assume erythropoiesis with *relative iron deficiency* (21).

Increased levels of hypochromic erythrocytes can also be observed in healthy patients having been treated with erythropoietin as a consequence of a number of autologous blood donations (22). However, this is not the case with patients having hereditary haemochromatosis and treated in the same way (23). These erythrocyte indices are really helpful in monitoring iron status and in identifying the need for iron supplementation during erythropoietin therapy in case of anaemia caused by chronic renal failure (24).

However, hypochromic erythrocytes also appear in patients with high levels of reticulocytes and young erythrocytes. Therefore, the usefulness of this parameter has to be critically reviewed (25, 26, 27).

4. Reticulocyte Indices - a “new dimension” for evaluating the erythropoietic iron status?

■ Reticulocyte number

18-36 hours prior to their final maturation, reticulocytes are released from the bone marrow into the peripheral blood. Consequently, they represent a "real-time" system for direct determination of the current status of erythropoiesis. However, it has to be understood that changes in the total concentration of reticulocytes *simply reflect the release* of more immature precursor stages and not a true increase of *real blood cell formation* in bone marrow (29, 30, 31, 32).

Some recent clinical studies have suggested evaluating the reaction to erythropoietin treatment by *measuring haemoglobin* and the *number of reticulocytes* 4 weeks after start of therapy. In this context, an increase in haemoglobin concentration of 1 g/dl or, in reticulocyte concentration by > 40 x 10⁹ /L, respectively, is indicative for a patient reaction to the treatment (33, 34).

■ The haemoglobin content of reticulocytes (CHr)

By means of newer haematology systems, it is possible to measure reticulocyte parameters like their average cell volume (MCVr), their haemoglobin concentration (CHCMr) and, (as a mathematical product of these two parameters for each cell measured (CVr-n x CHCr-n)) their haemoglobin content (CHr).

In a number of clinical studies the last-mentioned parameter, in particular, has proven to be useful in anaemia differentiation and in monitoring erythropoietin therapy.

For patients undergoing dialysis, sensitivity was 100 % for detecting relative iron deficiency, with a false-positive rate of 20 % (specificity 80%). The results have been more accurate than the parameters for serum ferritin, transferrin saturation and hypochromic erythrocytes, which have been examined in parallel.

It has been possible to show in other studies that a base value of 28 pg haemoglobin per single reticulocyte (CHr) yields a sensitivity of 78 %, with a specificity of 71 %, for detecting a *functional iron-deficiency anaemia*. Compared with the 50 % (sensitivity) and 39 % (specificity) values obtained for the clinical chemistry parameters measured at the same time, this represents a substantial improvement (35).

In case of patients undergoing dialysis simultaneously receiving iron administered intravenously during erythropoietin therapy, a significant increase in CHr was observed. Consequently, this means that this parameter is an early indicator of iron-deficient erythropoiesis (36), even with serum ferritin or transferrin saturation showing normal values (37). Illustrations are given in Figures 4-6.

In paediatrics, as well, this reticulocyte parameter has proven to be the best indicator for iron deficiency in children (38).

■ *IRF Immature Reticulocyte Fraction*

This parameter available on some flow haematology systems, detects the fraction of immature reticulocytes. For users of earlier systems, the following equation provides a reference point: $IRF = HFR + MFR$. However, gating algorithms for IRF have been further refined and adapted in relation to earlier “immature reticulocyte” parameters.

IRF clearly indicates the status of erythropoiesis, as well as direct erythropoietic activity but, with less sensitivity towards identification of a *functional iron deficiency* (39, 40).

5. The soluble transferrin receptor (sTfR)

The Tf-receptor, located on the cell membrane of reticulocytes, is also known as CD-71 marker in flow cytometry. Shortly after exclusion of the cell nucleus from the normoblast, a high concentration of cellular RNA remains. At the same time, the Tf-receptor is highly expressed. Both is required to transfer sufficient amounts of iron (bound to **transferrin**) into the red blood cells and ultimately to synthesise the necessary haemoglobin (using the remaining RNA). Thus, 20 to 30 % of the entire cellular haemoglobin in erythrocytes is synthesised during the last few days of maturation (41). In fact, this represents an inverse proportionate relationship between cellular nucleic acid (RNA/DNA) and the cellular haemoglobin content of a reticulocyte. In mature erythrocytes, the amount of nucleic acid (NA) is minimal (negligibly small), whereas the content of haemoglobin is at its maximum.

Therefore, it is conceivable that, in case of the red cell precursor both, the amount of haemoglobin (CHr), as well as, the amount of nucleic acids, equally provide information about the erythropoietic activity and its development over time. This is confirmed by means of new parameters available from the fluorescence flow channels of the new Sysmex XE-2100.

Serum ferritin may be the most sensitive and most specific parameter for iron status, *as long as there is still sufficient iron in the iron stores*. In case of an iron-deficient erythropoiesis, as for example in functional iron-deficiency anaemia, sTfR was found to be the more sensitive parameter (42). Both an increased erythropoiesis, as well as an iron deficiency, will produce an increase in sTfR (43, 44).

The clinical study described in the following section has been carried out by Prof. Sam Machin and his co-workers at University College Hospital, London (UCLH). sTfR was used as a reference method in order to evaluate the clinical and predictive value of the new red blood cell – and reticulocyte parameters. In this study, the authors conclude that the use of sTfR is limited for detecting functional iron deficiency, as well as for measuring the erythropoietic reaction to erythropoietin. STfR, however, it is a more sensitive indicator for iron deficiency independent from other influencing parameters such as inflammations, liver disorders, hormone status and sex (28).

6. New RBC- and Reticulocyte-Fluorescence-Parameters with the Sysmex XE-2100

The latest haematology system from Sysmex is the first and only analyser capable to differentiate and characterise all types of blood cells using fluorescence flow cytometry. This has also resulted in new research parameters in addition to parameters of the red blood cell series in the reticulocyte channel. After fluorescence staining by means a DNA/RNA-specific polymethine fluorochrome, the sample is characterised by three fold. Cells passing the analyser flow cell are excited by a 633 nm diode-laser and generate a forward scatter, a side scatter and a fluorescence signal. Figure 7 provides a schematic illustration of the principle.

The assumption was, that forward scatter of both, the erythrocytes, as well as, reticulocytes are depending on both, cell size (volume) **and** cell content (essentially haemoglobin and RNA/DNA). The most frequent bins for the distribution of the cell populations in the XE-2100 reticulocyte channel on the y-axis of the scattergram results in two new parameters:

- FS-e = forward scatter erythrocytes and
- FS-r = forward scatter reticulocytes.

In both cases, it simply represents the average of forward scattered light intensity for from erythrocytes (e) as well as reticulocytes (r). Figure 8 demonstrates the principle of cell distribution and the calculation of the respective parameters.

It can be assumed that the position of these populations, especially of reticulocytes, changes significantly with change in erythropoiesis.

Fig. 7: Generating and Measuring the New XE-2100 Parameters FS-e and FS-r

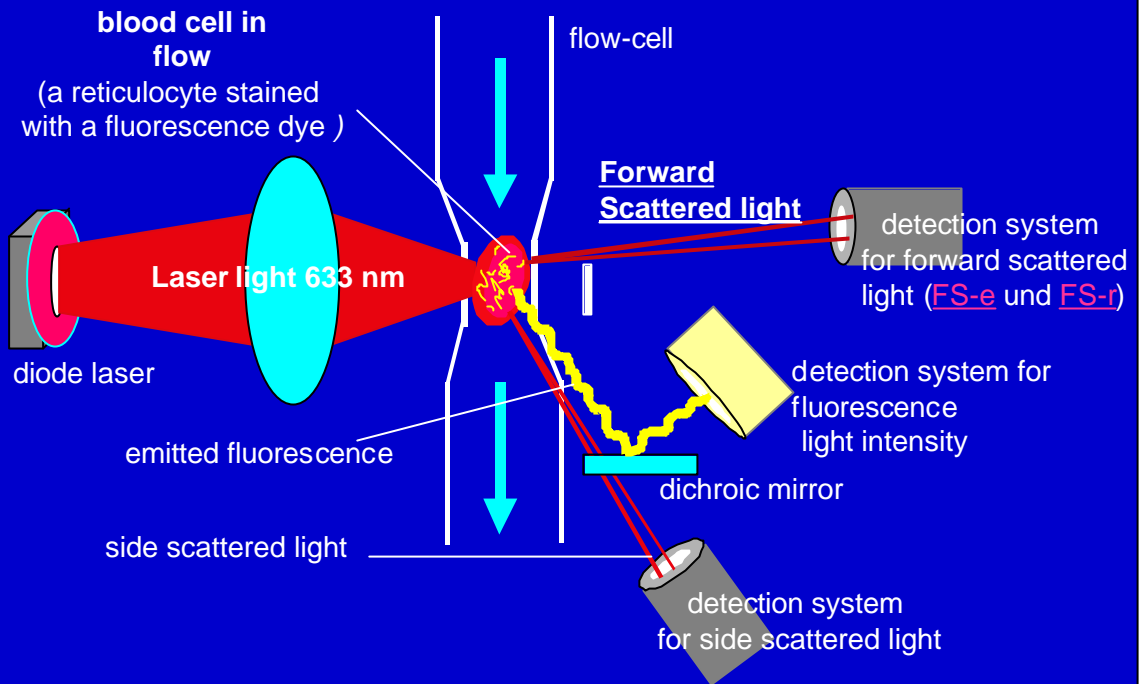
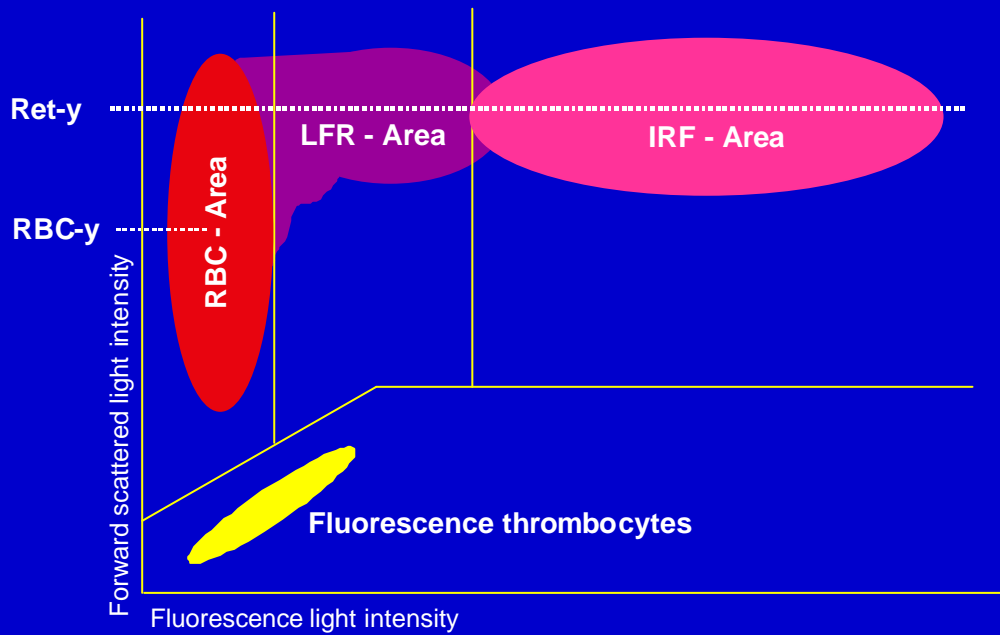


Fig. 8: Position of the New Red Cell Fluorescence Parameter in the Scattergram of XE-2100



Reticulocyte Channel of SYSMEX XE-2100

Thus the indirect measurement of erythropoietic activity is equivalent to the observations as described above for the parameters “% hypochromic erythrocytes” (corresponds to FS-e), and “CHr” (corresponds to FS-r).

This has been confirmed impressively by the study of Machin et al. at UCLH, London.

7. FS-r and FS-e correspond to the parameters CHr (reticulocyte haemoglobin content) and % hypo (percentage of hypochromic erythrocytes):

In total a group of 245 patient samples were examined. This group consisted of

- 40 healthy males (to determine the normal ranges)
- 50 samples from patients with iron deficiency
- 90 samples from patients with kidney disease prior to dialysis and CAPD, respectively
- 65 samples from patients undergoing long-term dialysis.

All samples were examined for the following parameters:

- CHr (Bayer Advia 120)
- % hypo (Bayer Advia 120)
- Ret-y (= FS-r) on Sysmex XE-2100
- RBC-y (= FS-e) on Sysmex XE-2100
- sTfR (Bio-stat test on Cobas Mira).

In addition, 70 samples were analysed:

- 20 samples from patients with iron deficiency
- 50 samples from patients with kidney disease.

With the exception of CHr, the same parameters have been determined as for the first cohort of samples (with the H*2 of Bayer, determination of CHr is not possible).

■ **Results of the UCLH Study for FS-e and FS-r**

Table 1 illustrates the normal ranges and the ranges defined as iron deficiency for CHr, % hypo, RBC-y (FS-e), Ret-y (FS-r) and sTfR.

TABLE 1: Normal ranges and ranges for iron deficiency

NORMAL RANGES					
	sTfR [mg/L]	CHr [pg]	% hypo	FS-e	FS-r
Mean / Average	1,4	31,7	1,0	175,6	184,4
Standard Deviation	0,3	1,57	0,99	3,35	5,28
2 x Standard Deviation	0,6	3,13	1,98	6,69	10,57
Minimum	0,93	28	0,1	168,7	171
Maximum	2,29	36,3	4,8	183,8	196,3
IRON DEFICIENCY					
	sTfR [mg/L]	CHr [pg]	% hypo	FS-e	FS-r
Mean / Average	4,9	24,2	38,3	137,6	141,6
Standard Deviation	3,26	2,14	19,07	15,63	18,76
2 x Standard Deviation	6,53	4,28	38,14	31,26	37,51
Minimum	1,56	19,3	4,3	93,6	86,4
Maximum	19,1	28,8	80,8	161,7	171,1

Significant differences exist between the normal ranges and the iron-deficiency ranges for the first four parameters. Only for sTfR an overlap between the normal range and the iron-deficiency range occurs which is an impediment for the use of this parameter in the unambiguous differentiation of iron-deficiency anaemia. Indeed, 9 out of 70 patients with iron deficiency had normal sTfR values.

Excellent correlations have been obtained for all samples between FS-r and CHr, as well as, between FS-r and % hypo ($r = 0.94$ as well as 0.91). Figure 9a & 9b.

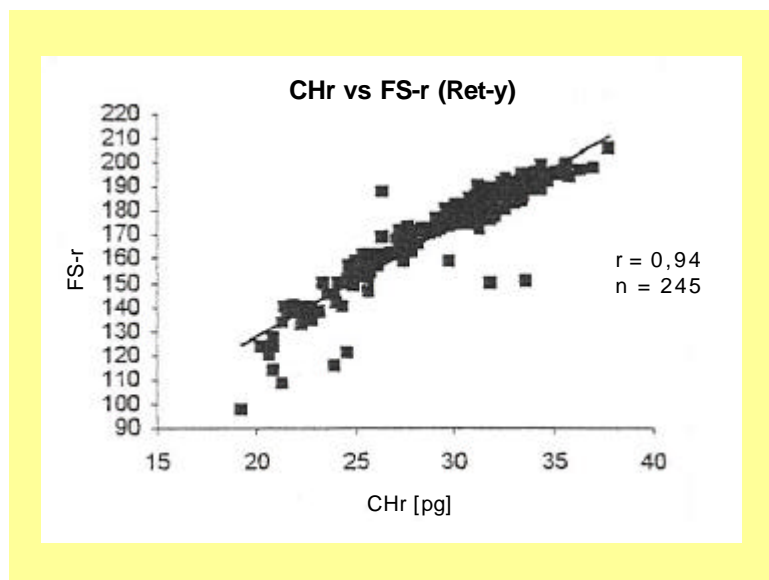


Figure 9a: Correlation of the reticulocyte haemoglobin concentration (CHr) and FS-r for all patient-groups.

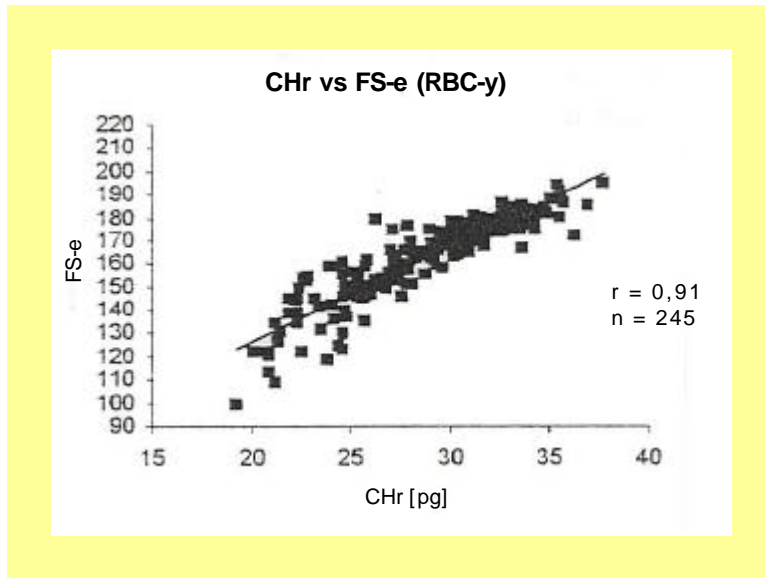


Figure 9b: Correlation of reticulocyte hemoglobin concentration (CHr) and FS-e for all patient-groups.

Correlation of % hypo with FS-e and FS-r ($r = 0.84$ and $r = 0.81$) was somewhat better than that of CHr ($r = 0.75$). Figure 10 a, b & c

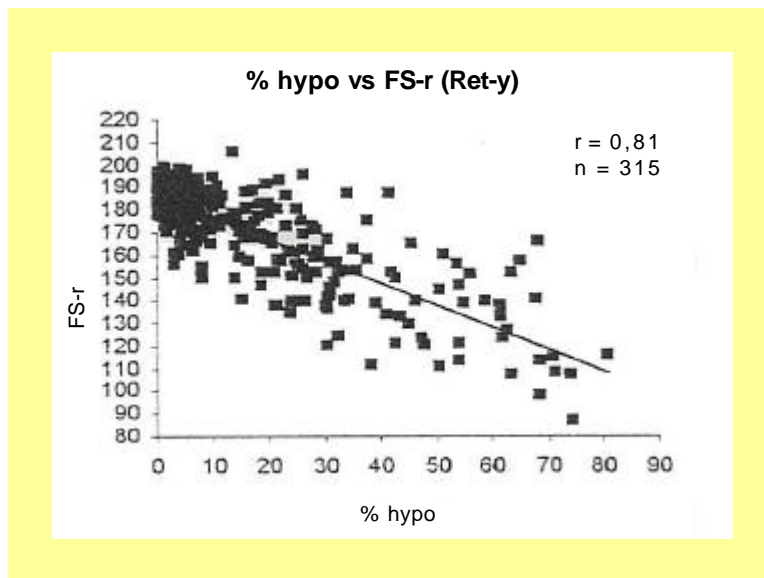


Figure 10a: Correlation of % hypo and FS-r for all patient-groups

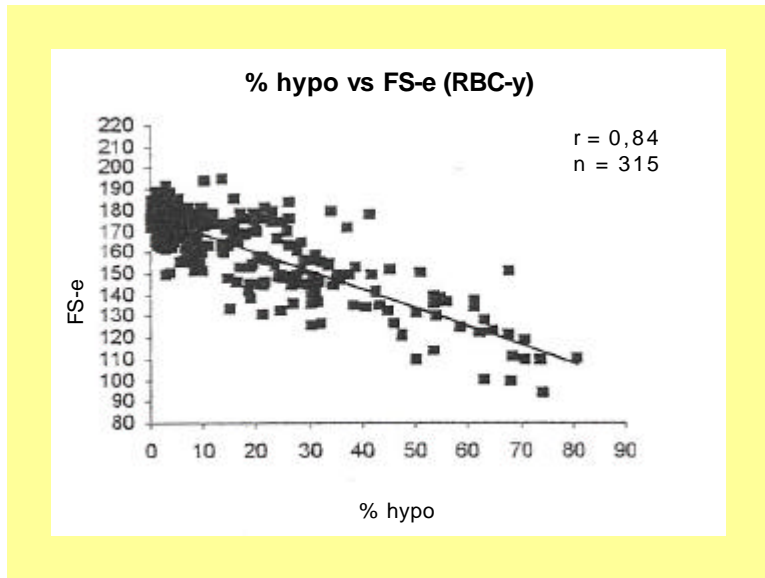


Figure 10b: Correlation of % hypo and FS-e for all patient-groups

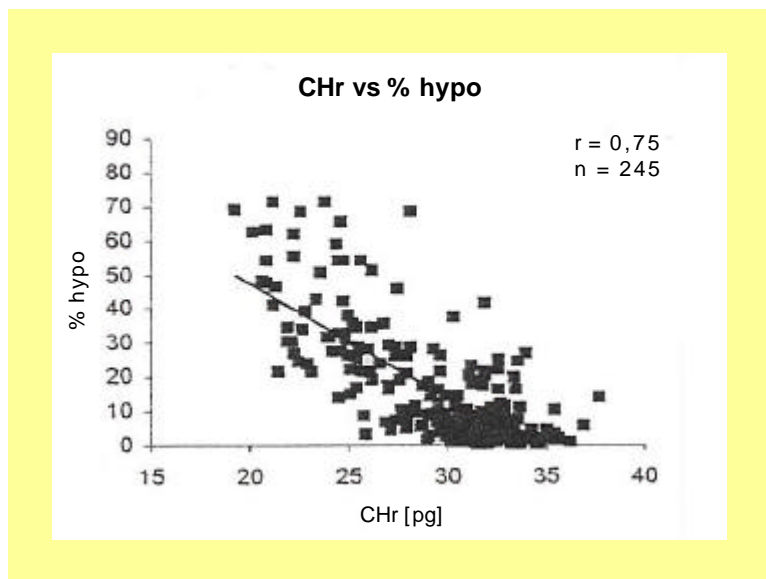


Figure 10c: Correlation of % hypo and CHr for all patient-groups

When comparing CHr and FS-r, three samples show a normal CHr with a simultaneously abnormally reduced FS-r. Two of these three patients also showed haematological illnesses in addition to renal failure requiring dialysis: one presented a myeloma, the other AML. This means that the XE-parameter was more sensitive than the corresponding CHr for these cases. A similar situation was found when comparing % hypo with FS-e.

Comparisons of sTfR with RBC/Reti indices for the two systems also showed significant correlations of $r = 0.68$ for both parameters, FS-e and FS-r.

When comparing “% hypo” to sTfR, r is only slightly reduced (0.66). With CHr, however, the correlation is of marginal value ($r = 0.55$). This finding raises the question if sTfR is suitable as an indicator of iron deficiency, as well as of the erythropoietic status (figure 11a, b, c & d). Additionally, it underlines a better sensitivity of the XE-2100 parameters towards detection of iron deficiencies as compared to the Bayer system parameters.

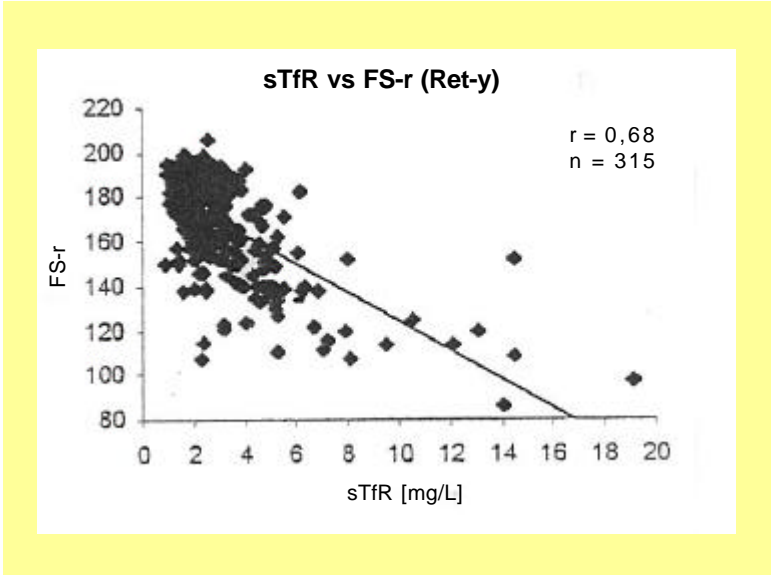


Figure 11a: Correlation of sTfR and FS-r for all patient-groups

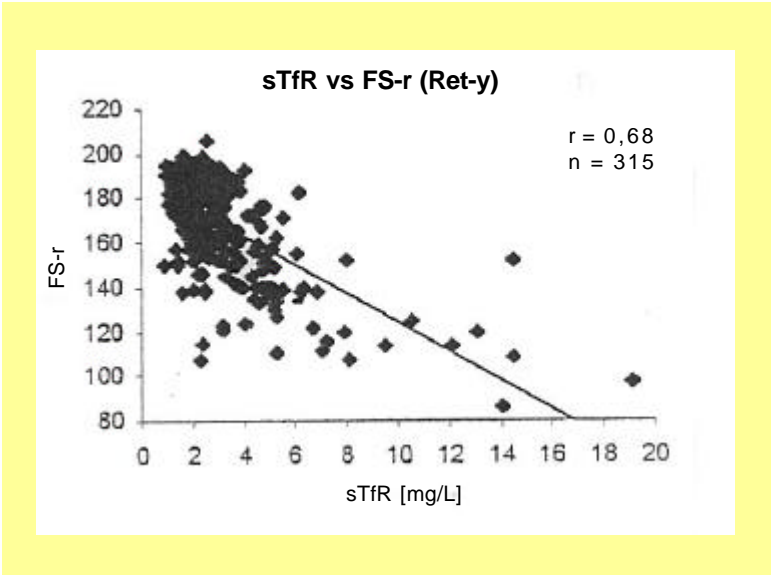


Figure 11b: Correlation of sTfR and FS-e for all patient-groups

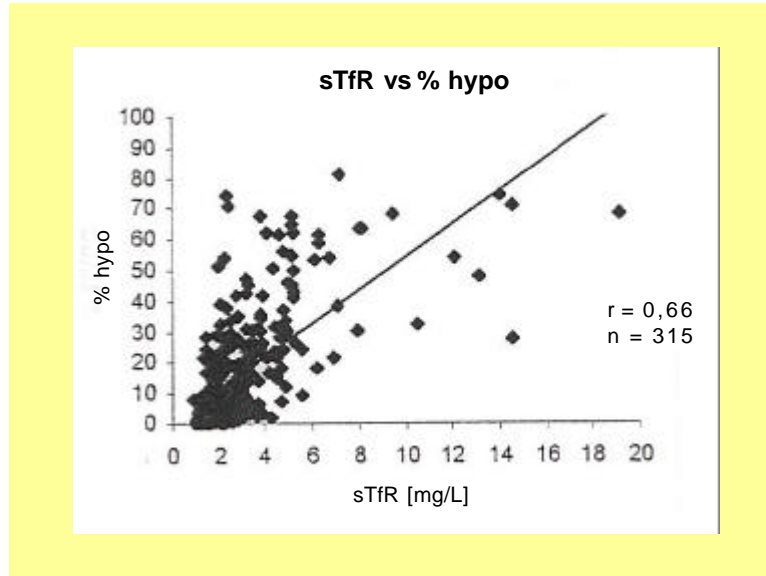


Figure 11c: Correlation of sTfR and % hypo fro all patient-groups

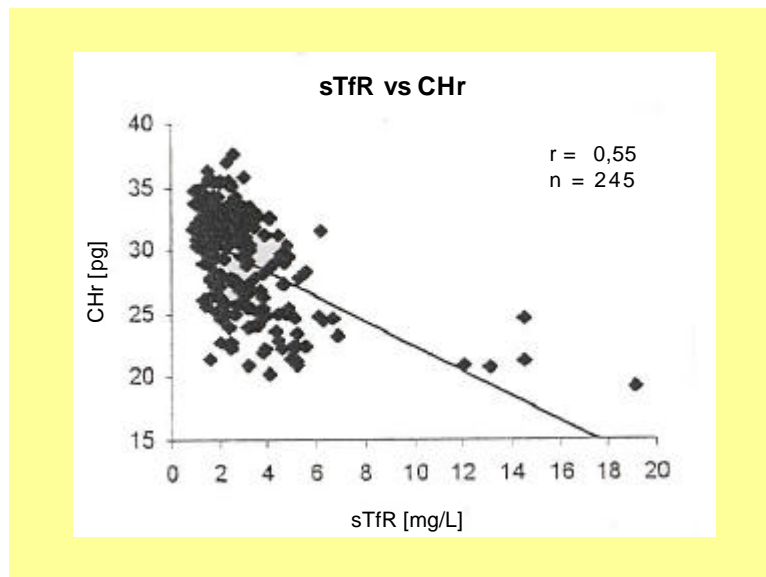


Figure 11d: Correlation of sTfR and CHr for all patient-groups

Assuming a sTfR-concentration of 2.29 mg/L as the limit for iron deficiency, (see Table 1; the upper limit of defined normal range) and analysing the accuracy of the remaining parameters in relation to that, a true positive as well as a true negative predicament in approximately 70% of the samples is the result.

The parameters "% hypo" shows less false-negatives than CHr, FS-e and FS-r. On the other hand, however, there are more false-positives. CHr, FS-r and FS-e show approximately the same number of false-negatives.

FS-e shows more false-positives than CHr and FS-r – certainly in analogy with % hypo.

A remarkable agreement has been found between CHr and FS-r. All "false" (in comparison with sTfR), for both parameters are from identical samples! Table 2 illustrates the results.

CHr	% hypo
„True Pos“ or „TrueNeg“ = 69,6%	„True Pos“ or „True Neg“ = 73,2%
„False Neg“ = 22,2%	„False Neg“ = 7,7%
„False Pos“ = 8,2%	„False Pos“ = 19,1%
„False“ Results = 30,4%	„False“ Results = 26,8%
FS-r	FS-e
„True Pos“ oder „True Neg“ = 70,0%	„True Pos“ or „True Neg“ = 68,0%
„False Neg“ = 21,5%	„False Neg“ = 19,4%
„False Pos“ = 8,5%	„False Pos“ = 12,6%
„False“ Results = 30,0%	„False“ Results = 32,0%

TABLE 2: Possibility of detecting iron-deficiency anaemia using the parameters CHr, % hypo, FS-r and FS-e (definition of iron deficiency: sTfR > 2.29 mg/L).

An additional comparative analysis (data not shown) with serum ferritin yielded results not very revealing (for example, no correlation between sTfR and ferritin). This is not at all remarkable since ferritin is an acute-phase protein increasing considerably in chronic diseases.

8. Discussion

The measurement of forward scattered light for RBC and reticulocytes in the reticulocyte channel of XE-2100 has been proven to be of clinical use. In addition to cell size it could be evidently shown that, also the cellular content of Hgb *and* nucleic acid residues do impact forward scattered light intensity.

Lower cellular haemoglobin content results in significantly lower scattered light intensities for the cell populations under consideration. With iron deficiency, FS-r drops from 184.4 (normal value) to 171 (-25%). FS-e for erythrocytes decreases from 175.6 (normal value) to 168.7 (-12%). This is in accordance to the identical findings for the previously described parameters “CHr” and “% hypo” of the Bayer systems.

Compared to the “reference method” of sTfR, the XE-2100 parameters yielded better correlations than “CHr” and “% hypo”. CHr showed the lowest correlation coefficient with $r = 0.55$.

However, the equivalence of both, the measured results and the resulting clinical consequence based on CHr and FS-r (using the XE-2100) is remarkable. This led Machin and his team to conclude that, independent from the role of “% hypo” and “CHr” in surveillance and monitoring of iron status and erythropoietic activity, *FS-r and FS-e can be used in exactly the same way* (45).

With those new parameters on the Sysmex XE-2100, an extended red blood cell diagnostics on native cells - without chemically changing their conformation - seems to be possible now.

Detection of functional iron deficiency, as well as monitoring or management of dialysis patients requiring erythropoietin (with or without iron supplement), is likewise possible. More extensive clinical studies on these issues are currently in progress.

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